

Original Article

Comprehensive pan-cancer analysis reveals VSIR as a candidate immunologic, diagnostic, and prognostic biomarker

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Abstract: Objectives: Being a checkpoint, the expression level of V-set immunoregulatory receptor (VSIR) serves as an indicator of the extent of immunosuppression. Our objective was to undertake a pan-cancer analysis to examine the expression, genetic alterations, prognosis, and immunologic features associated with VSIR. Methods: The Cancer Genome Atlas (TCGA), Genotype-Tissue Expression (GTEx), GEPIA2, UALCAN, OncoDB, Human Protein Atlas (HPA), STRING, DAVID, cell culture, clinical sample collection, and reverse transcription quantitative polymerase chain reaction (RT-qPCR) were used. Results: This study comprehensively assessed VSIR across 33 cancers using TCGA and GTEx databases. Differential expression analysis revealed elevated VSIR in several cancers, notably in cholangiocarcinoma, esophageal carcinoma, kidney renal cell carcinoma, and liver hepatocellular carcinoma, while decreased expression was observed in various others. Prognostic analysis highlighted its significant association with reduced overall survival (OS) in ESCA and LIHC. Investigation into cancer stages demonstrated a correlation between VSIR expression and stage in ESCA and LIHC. Promoter methylation analysis indicated decreased VSIR methylation levels in tumors, implicating a role in oncogenesis. Furthermore, subcellular localization predictions, Tumor Mutational Burden (TMB), and Microsatellite Instability (MSI) correlations revealed intriguing insight into VSIR's function. Notably, a positive correlation was identified between VSIR expression and various immune cells in both cancers. Protein-protein interaction (PPI) network construction and gene enrichment analysis elucidated VSIR-associated dysregulated pathways, emphasizing its possible involvement in diverse pathways. Finally, experimental validation using LIHC clinical samples and cell lines confirmed elevated VSIR expression, supporting its oncogenic role. Conclusion: Collectively, these findings present a comprehensive understanding of VSIR's diverse roles and potential clinical implications in ESCA and LIHC.

Keywords: VSIR, pan-cancer, biomarker, prognosis

Introduction

Cancer stands as the leading global cause of mortality [1, 2]. Enhancing our understanding of carcinogenesis and tumor progression is

imperative to broaden the spectrum of therapeutic possibilities for malignancies, primarily by identifying oncogenes. Over the past decade, the availability of extensive and multi-omics cancer datasets, including The Cancer Genome

VSIR as a pan-cancer marker

Atlas (TCGA) [3], has facilitated pan-cancer analyses, allowing for comprehensive insight into the intricate landscape of cancer.

Furthermore, current cancer treatment encompasses conventional anti-cancer medications, immunotherapy, and approaches such as chimeric antigen receptor T cell (CAR-T) therapy [4, 5]. Nevertheless, the efficacy of traditional anti-cancer drugs is compromised by anti-cancer drug tolerance (ADT), limiting their full potential. Additionally, tumor cells can evade immune responses by activating pathways within the tumor microenvironment (TME) that hinder effective immune reactions [6, 7]. The immune escape pathway of tumors is significantly influenced by immune checkpoints, and the inhibition of these checkpoints holds promise as a novel avenue in cancer treatment. Presently, numerous studies have unraveled the mechanisms and potentials of key immune checkpoints, including programmed cell death protein 1 (PD-1), in the realm of cancer treatment [8-10]. Immune checkpoint suppression (ICS) is emerging at the forefront of cancer treatment.

V-Set Immunoregulatory Receptor (VSIR), also recognized as VISTA, C10orf54, PD-1H, B7H5, GI24, PP2135, or SISP1, belongs to the immunoglobulin superfamily and is a type 1 transmembrane protein [11]. The cytoplasmic tail domain of VSIR contains two possible protein kinase C binding sites and proline docking residues, suggesting its capability to function as either a receptor or ligand in cellular processes [11]. VSIR can be expressed by multiple cell types, encompassing cancer cells, neutrophils, monocytes, macrophages, dendritic cells (D.C.s), and T cells in both humans and mice [12, 13]. Its involvement in various physiological and pathological processes has been documented, including the regulation of peripheral tolerance, induction of T cell activation and differentiation, and mediation of tumor immunity [14, 15]. Consequently, evidence suggests that VSIR could serve as a target for tumor immunotherapeutic intervention within the tumor microenvironment (TME). However, the deregulation of VSIR across various cancers, such as lung, colorectal, and esophageal cancers [16-18], has been reported in only a few studies to date. Nevertheless, the regulatory pan-cancer role of VSIR in humans has not been thoroughly elucidated. To address this gap, a

comprehensive analysis covering 33 different cancer types was conducted to further investigate the association between VSIR expression and prognosis.

Methods

Expression analysis of VSIR in view of pan-cancer

The VSIR expression profiles across 33 cancer types were sourced from the Cancer Genome Atlas (TCGA) database. To facilitate comparisons, both normal and cancer tissue samples from the TCGA and the Genotype-Tissue Expression (GTEx) portal databases were incorporated, and the expression levels of VSIR were normalized using Log_2 transformation in these datasets.

Prognostic value of VSIR in view of pan-cancer

GEPIA2 (Gene Expression Profiling Interactive Analysis 2) is a web-based tool providing comprehensive analyses of RNA sequencing expression data from tumors and normal samples [19]. It enables users to explore gene expression patterns, perform survival analyses, and compare expression levels across various cancers, enhancing insights into the molecular landscape of diverse diseases. In the present study, GEPIA2 was used to analyze the prognostic value of VSIR gene in pan-cancer.

VSIR expression across different clinical variables of cancer patients

Leveraging TCGA data, GEPIA2 provides a comprehensive platform for analyzing gene expression, survival, and clinicopathologic features across different cancer types. In the present study, GEPIA2 was further used to analyze VSIR expression across different cancer stages of the selected cancer types.

Promoter methylation analysis

OncoDB is a comprehensive cancer database providing valuable information on genetic alterations in various cancers [20]. It facilitates in-depth exploration of oncogenes, aiding researchers in understanding cancer molecular profiles. In the present study, OncoDB database was used to analyze VSIR promoter methylation level in specified cancers.

VSIR as a pan-cancer marker

Mutation analysis of VSIR

cBioPortal is a powerful cancer genomics web resource that enables the exploration of large-scale cancer genomic datasets [21]. Researchers can analyze genetic alterations, visualize data, and interpret complex cancer genomics. This database was used in the current study to analyze the mutational spectrum of VSIR across specified cancer types.

Human Protein Atlas (HPA) analysis

Human Protein Atlas (HPA) is a valuable resource offering extensive insights into human proteins' spatial distribution in cells, tissues, and organs [22]. Utilizing immunohistochemistry and transcriptomics data, HPA enables researchers to explore protein expression profiles, aiding in understanding cellular functions and their implications in various physiological and pathological contexts. In the present study, HPA database was used to analyze VSIR protein structure and subcellular localization.

Correlation of VSIR expression with mutational tumor heterogeneity

The association between VSIR and tumor mutation burden (TMB) or microsatellite instability (MSI) was evaluated through Pearson correlation analysis. Subsequently, a radar plot illustrating these correlations was generated using the FMSB R package (v0.7.5).

Correlation between VSIR expression and immune cells

TIMER (Tumor Immune Estimation Resource) provides comprehensive insights into immune infiltrates' abundance across diverse cancer types [23]. Combining genomic data from TCGA, TIMER enables researchers to explore the intricate interplay between tumors and the immune microenvironment. In this study, TIMER database was used to analyze correlation between VSIR expression and various immune cells.

Protein-protein interaction (PPI) network construction and gene enrichment analysis

The STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database facilitates the exploration of protein-protein interac-

tions, offering a comprehensive platform to analyze and visualize molecular networks [24]. In the present study, this database was used to construct the PPI of VSIR-associated proteins.

DAVID (Database for Annotation, Visualization, and Integrated Discovery) is a bioinformatic resource offering functional annotation and analysis of gene lists [25]. It aids in elucidating the biological significance of gene sets by integrating diverse annotation sources, facilitating a comprehensive understanding of biological pathways and processes. In the present study, DAVID was utilized for the gene enrichment analysis of VSIR-associated genes.

Clinical sample collection

A total of 23 LIHC tissue samples, along with corresponding controls, were obtained from patients undergoing surgery at Nishtar Hospital, Multan, Pakistan. Each sample underwent microscopic examination to ensure a tumor cell content of $\geq 80\%$ before being promptly preserved in liquid nitrogen. Ethical approval for this study was granted by the institutional review boards, and written informed consent was obtained from all participating patients.

Cell culture

A single control cell line (LO2) and three hepatocellular carcinoma (HCC) cell lines, including Hep-G2, SNU-387, and Huh-7, were procured from the American Type Culture Collection (ATCC) and China Cell Bank. The cells were maintained in DMEM medium (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, USA) and incubated at 37°C with 5% carbon dioxide.

RNA isolation and RT-qPCR

Tissue samples and cell lines were lysed using TRIzol reagent (Life Technologies, USA) for total RNA extraction. Then, purity and concentration of RNA was measured by spectrophotometric value and A260/A280 ratio. Reverse transcription of RNA was performed with the RevertAid™ First Strand cDNA Synthesis Kit (Life Technologies, USA). The iQ SYBR Green Supermix PCR kit (Bio-Rad) was employed for quantitative gene expression analysis on the Rotor-Gene 3000 system (Corbett Research). Gene expression levels were normalized to the housekeeping gene GAPDH, and relative mRNA

VSIR as a pan-cancer marker

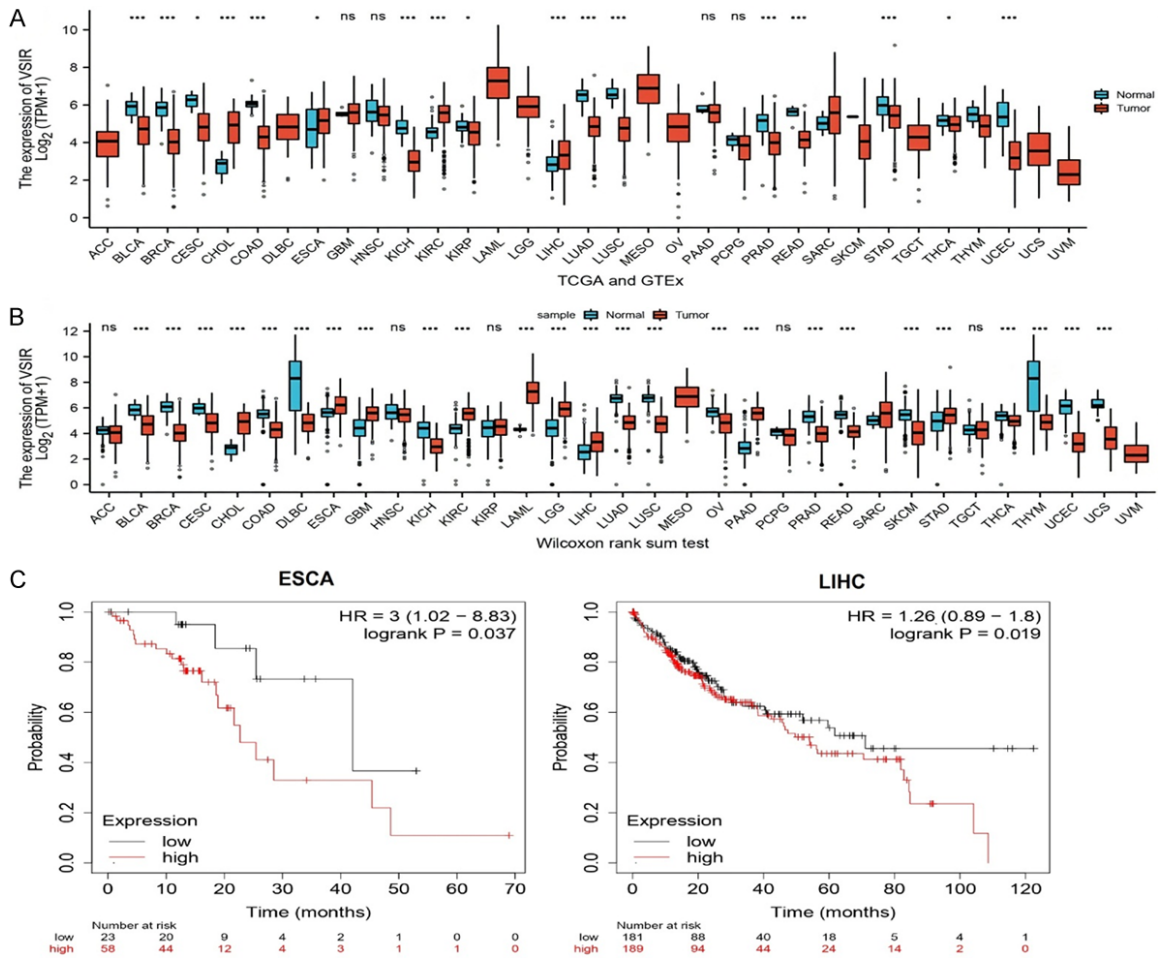


Figure 1. Expression and prognostic analysis of V-set immunoregulatory receptor (VSIR) in cancer patients. A. Pan-cancer expression pattern of VSIR using The Cancer Genome Atlas (TCGA) dataset. B. Pan-cancer expression pattern of VSIR across combined TCGA and Genotype-Tissue Expression (GTEx) datasets. C. Kaplan-Meier (KM) curves depicting the prognostic significance of VSIR in esophageal carcinoma (ESCA) and liver hepatocellular carcinoma (LIHC) patients. P -value < 0.05.

levels were determined using the comparative C_t ($2^{-\Delta\Delta C_t}$) method.

The primers were as follows: VSIR-F: 5'-AGATG-CACCATCCAAGTGTGTGG-3', VSIR-R: 5'-AGGCAG-AGGATTCTACGATGC-3', GAPDH-F: 5'-CTGGGC-TACTGAGCACC-3', GAPDH-R: 5'-AAGTGGTCG-TTGAGGGCAATG-3'.

Statistical analysis

Common statistical analyses such as the Student t-test were employed, while survival analysis utilized the log-rank test. The Spearman correlation analysis was applied to evaluate the association between VSIR expression and immune scores. The diagnostic value of VSIR was analyzed with the help of a Receiver

Operating Characteristic (ROC) curve. All computations were conducted using the R package, and significance was set at a p -value < 0.05.

Results

Expression analysis of VSIR in view of pan-cancer

Utilizing the TCGA dataset, we evaluated VSIR mRNA expression across 33 cancers. The absolute expression levels in normal and cancer tissues from TCGA, combined with GTEx database, are depicted in **Figure 1**. Results revealed significantly elevated VSIR mRNA expression in cholangiocarcinoma, esophageal carcinoma, kidney renal cell carcinoma, and liver hepatocellular carcinoma, while it exhibit-

VSIR as a pan-cancer marker

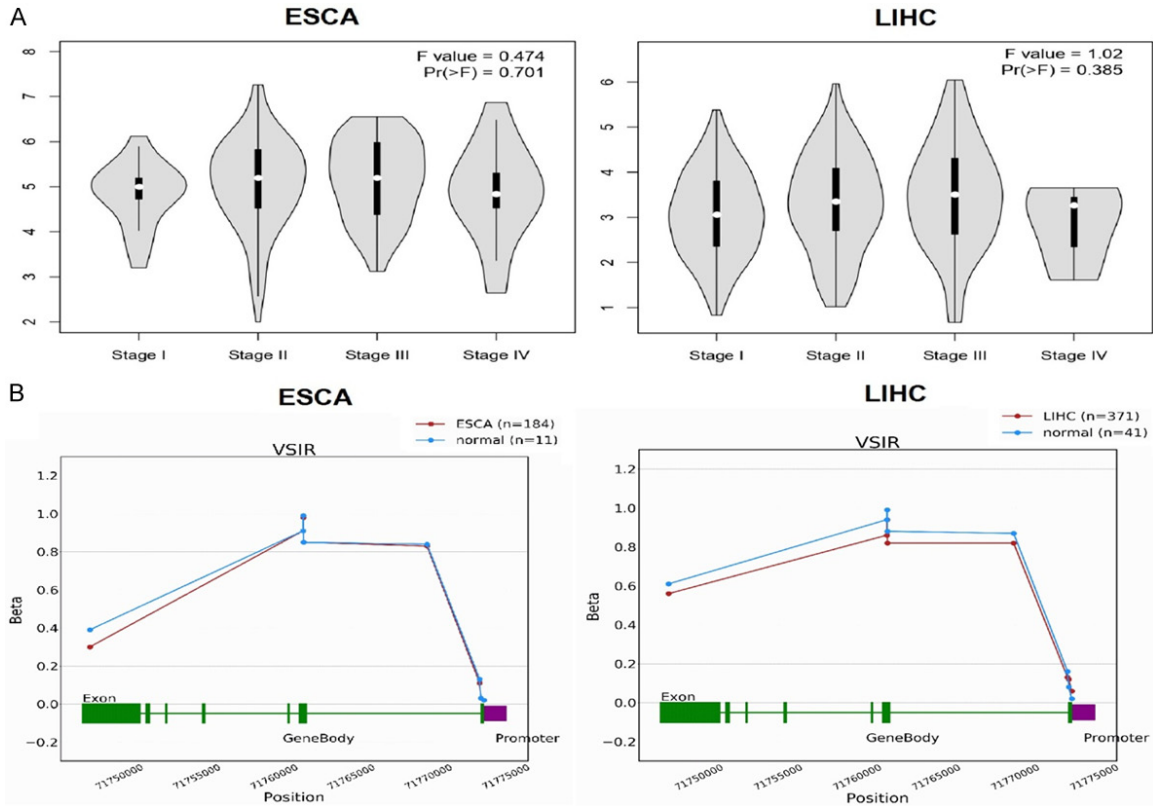


Figure 2. V-set immunoregulatory receptor (VSIR) expression pattern across diverse stages and includes promoter methylation analysis in esophageal carcinoma (ESCA) and liver hepatocellular carcinoma (LIHC) patients. A. VSIR expression across various stages of ESCA and LIHC. B. VSIR promoter methylation pattern in ESCA and LIHC samples. P -value < 0.05.

ed lower expression in various cancers (see Figure for abbreviations) including BLCA, BRCA, CESC, COAD, KICH, KIRP, LUAD, LUSC, PRAD, READ, STAD, THCA, and UCEC (**Figure 1A**). Furthermore, with the inclusion of the GTEx dataset, we expanded our analysis to encompass more VSIR mRNA expressions in normal tissues (**Figure 1B**). Examination of the TCGA-GTEx dataset revealed significantly elevated VSIR mRNA expression in CHOL, ESCA, KIRC, LIHC, GBM, LAML, LGG, PAAD, SARC, and UCEC (**Figure 1B**). Conversely, VSIR mRNA exhibited lower expression in BLCA, BRCA, CESC, COAD, DLBC, KICH, LUAD, LUSC, OV, PRAD, READ, THCA, THYM, UCEC, and UCS (**Figure 1B**).

Prognostic value of VSIR in view of pan-cancer

We conducted overall survival (OS) analysis across various cancer cohorts using GEPIA2. Elevated VSIR expression demonstrated a significant association with reduced OS specifically in two cancers: esophageal carcinoma (ESCA, p -value < 0.05) and liver hepatocellular

carcinoma (LIHC, p -value < 0.05) (**Figure 1C**). These findings suggest a crucial involvement of VSIR overexpression in the advancement and progression of ESCA and LIHC.

Correlation of VSIR with different stages and the analysis of promoter methylation level in esophageal and liver cancer

Furthermore, we examined the association between VSIR expression and distinct cancer stages (I, II, III, and IV) in ESCA and LIHC using the GEPIA2 database. The analysis revealed a significant correlation (p -value < 0.05) between VSIR expression and various cancer stages among ESCA and LIHC patients (**Figure 2A**).

Aberrant methylation is associated with oncogenesis, and variations in methylation patterns distinguish tumors from benign tissues [26-30]. Methylation can act as either a promoter or an inhibitor of tumor formation. Consequently, we examined differences in VSIR promoter methylation levels between tumors and adjacent normal tissues utilizing the OncoDB data-

VSIR as a pan-cancer marker

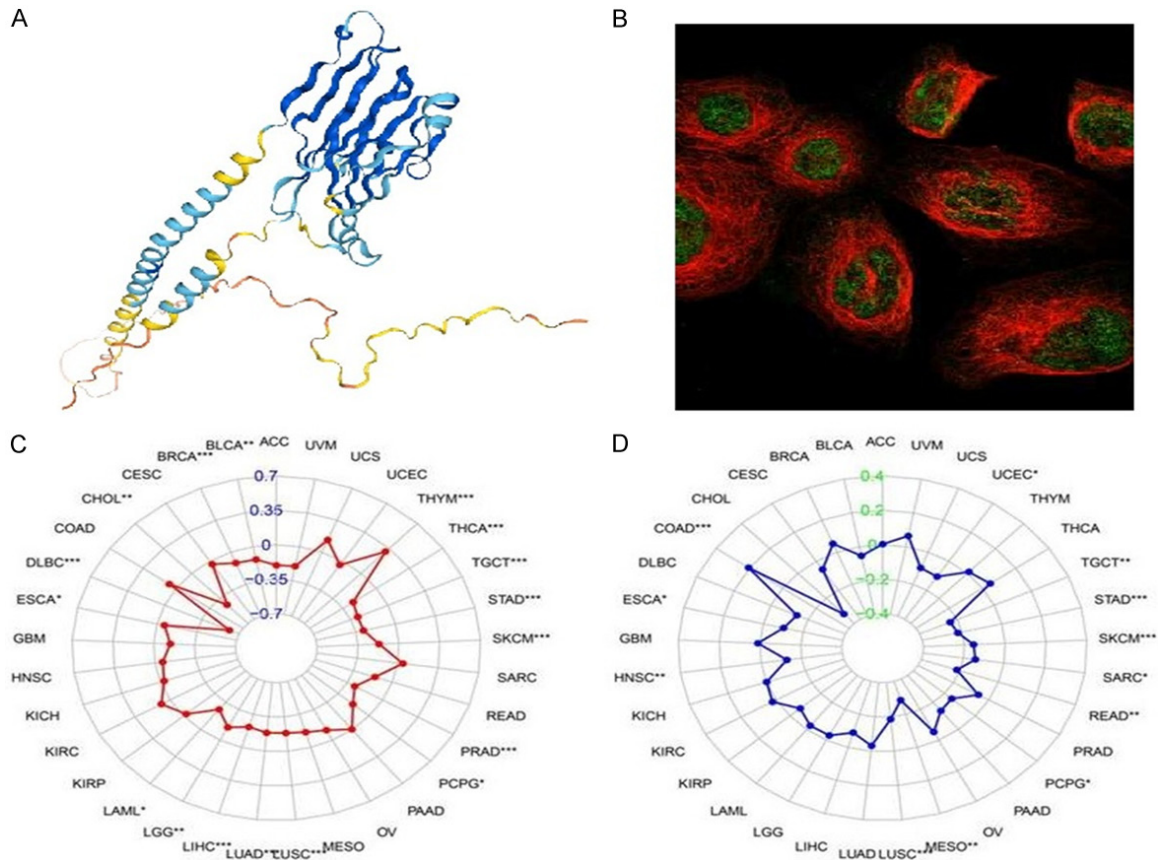


Figure 3. Subcellular localization prediction and correlation analysis of V-set immunoregulatory receptor (VSIR) with tumor mutational burden (TMB) and microsatellite instability (MSI). A, B. 3D structure and subcellular localization of the VSIR protein within the nucleosome. C. Correlation of VSIR with TMB. D. Correlation of VSIR with MSI. *P*-value < 0.05.

base. The analysis indicated a decrease in VSIR promoter methylation levels in ESCA and LIHC compared to their adjacent normal tissues (Figure 2B).

Sub-cellular localization prediction and TMB and MSI correlation with VSIR expression

Subcellular localization prediction analysis by HPA database showed that VSIR protein was mainly localized in the nucleoplasm (Figure 3A, 3B). Tumor mutational burden (TMB) is a novel and developing biomarker that correlates with immune checkpoint inhibitor sensitivity [31]. Our study shows that VSIR expression is negatively correlated with TMB in ESCA and LIHC (Figure 3C). Additionally, we looked into whether VSIR expression might be associated with microsatellite instability (MSI) in ESCA and LIHC. The results showed that VSIR expression was negatively correlated with MSI in ESCA and LIHC (Figure 3D).

Mutational analysis of VSIR in ESCA and LIHC

Genetic mutations are pivotal in disrupting the expression of crucial genes, contributing to dysregulation and promoting cancer development [32-34]. Utilizing the cBioPortal online database, we examined genetic mutations in VSIR across ESCA and LIHC samples from TCGA datasets (Figure 4). The findings revealed a 0% mutational frequency of VSIR in both ESCA and LIHC samples (Figure 4). In conclusion, these results underscore that VSIR remains unmutated in ESCA and LIHC.

Correlation between VSIR expression and immune cells

Tumor-infiltrating immune cells constitute the central elements within the tumor microenvironment, significantly influencing cancer initiation, progression, and metastasis [35, 36]. The TIMER database facilitated the examination of

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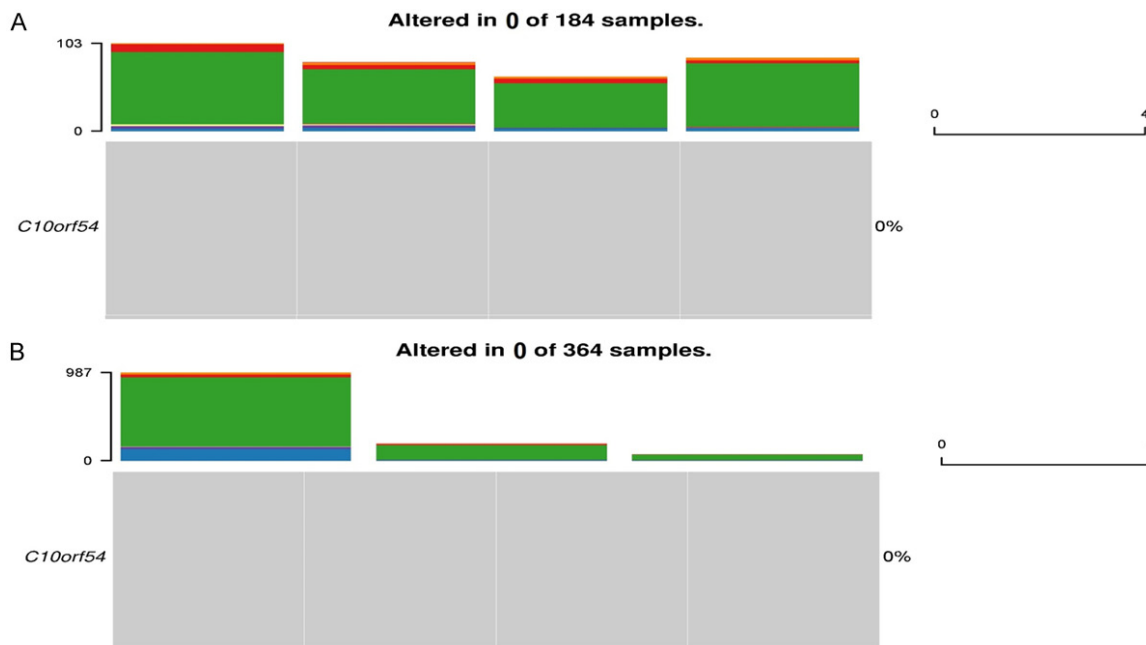


Figure 4. Mutation features of V-set immunoregulatory receptor (VSIR) in esophageal carcinoma (ESCA) and liver hepatocellular carcinoma (LIHC). A. Mutation features observed in ESCA. B. Mutation features identified in LIHC.

correlations between VSIR expression and various immune cells. The findings indicated a positive correlation between VSIR expression and B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells in ESCA (**Figure 5A**) and LIHC (**Figure 5B**). This collectively implies that the expression of VSIR likely contributes to the recruitment of these immune cells in the tumor microenvironment (TME).

Protein-protein interaction (PPI) network construction and gene enrichment analysis

Using the STRING tool, we first constructed the PPI of CTHRC1-binding proteins to identify a potential role of the CTHRC1 gene in tumor pathogenesis. The constructed PPI revealed that a total of 21 proteins interact with VSIR in tumor pathogenesis (**Figure 6A**). The findings of the GO analysis demonstrated that, in terms of Cellular Component (CC), the VSIR-associated proteins exhibited significant enrichment in “Pinosome, macropinosome, and heteromeric SMAD protein complex etc., terms” (**Figure 6B**). Furthermore, in the Molecular Functions (MF) analysis, the VSIR-associated proteins were predominantly associated with the “Sterol response element binding, disaccharide binding, and galactose binding etc., terms” (**Figure 6C**). Furthermore, the Biological Processes (BP)

of VSIR-associated proteins include “Positive reg. of cysteine-type endopeptidase activity involved in apo, positive reg. of transforming growth factor beta production, and reg. of mast cells degranulation etc., terms” (**Figure 6D**). To gain further insight into the VSIR-associated dysregulated pathways, a KEGG pathway analysis was conducted. The findings revealed that VSIR along with other interacting proteins was predominantly enriched in colorectal cancer, signaling pathways regulating pluripotency of stem cells, and gastric cancer signaling pathways (**Figure 6E**).

Validation of VSIR expression using LIHC clinical samples and cell lines

The RT-qPCR analysis was employed to assess VSIR expression in LIHC clinical samples and cell lines, delving into its functional implications. Relative to the normal control (LO2), LIHC cell lines (Hep-G2, SNU-387, Huh-7) exhibited elevated VSIR expression levels, with SNU-387 demonstrating the highest expression, warranting further investigation (**Figure 7A**). Concomitantly, LIHC tissue samples (n = 23) displayed significantly higher VSIR levels compared to normal controls (n = 23) (**Figure 7B**). The ROC curve underscored the diagnostic potential of up-regulated VSIR (**Figure 7C**). These findings

VSIR as a pan-cancer marker

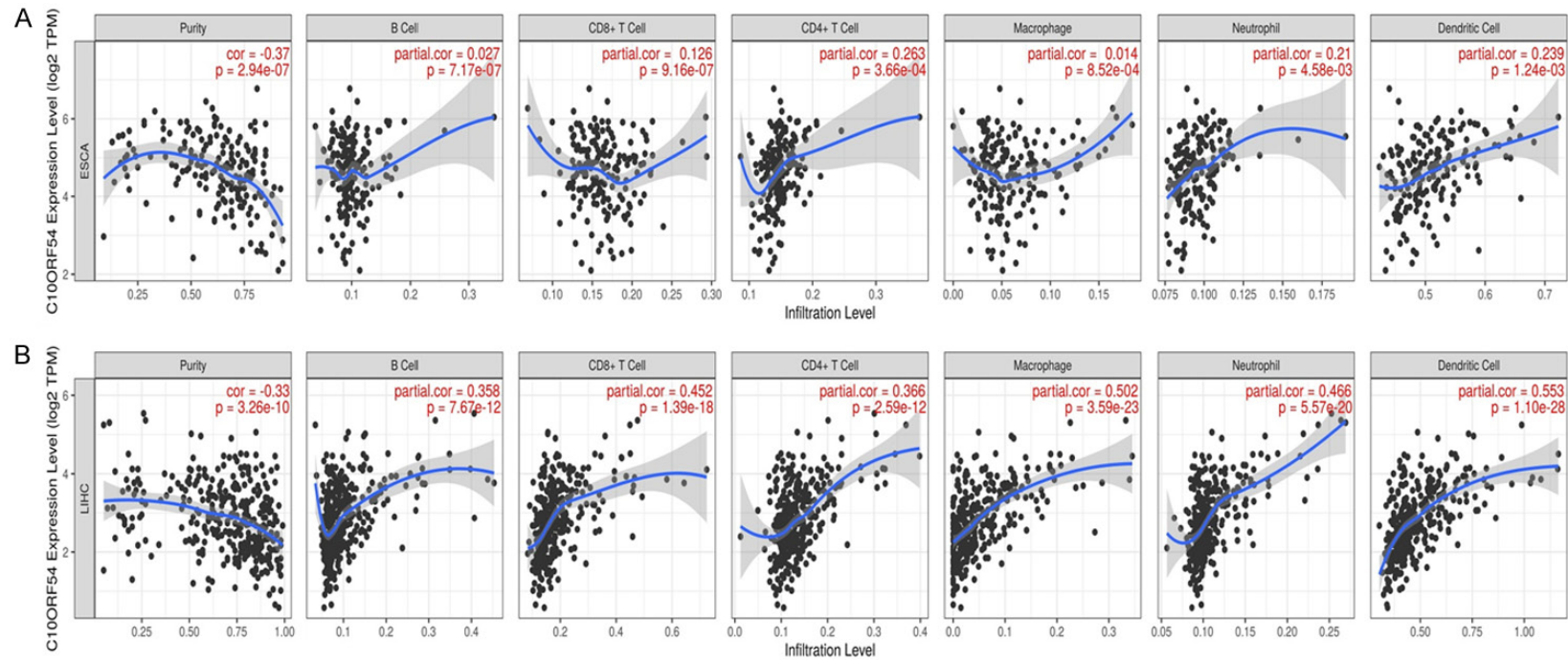


Figure 5. Correlation between V-set immunoregulatory receptor (VSIR) expression and immune infiltration. A. Correlation of VSIR expression with immune infiltration levels in esophageal carcinoma (ESCA). B. Correlation of VSIR expression with immune infiltration levels in liver hepatocellular carcinoma (LIHC). *P*-value < 0.05.

VSIR as a pan-cancer marker

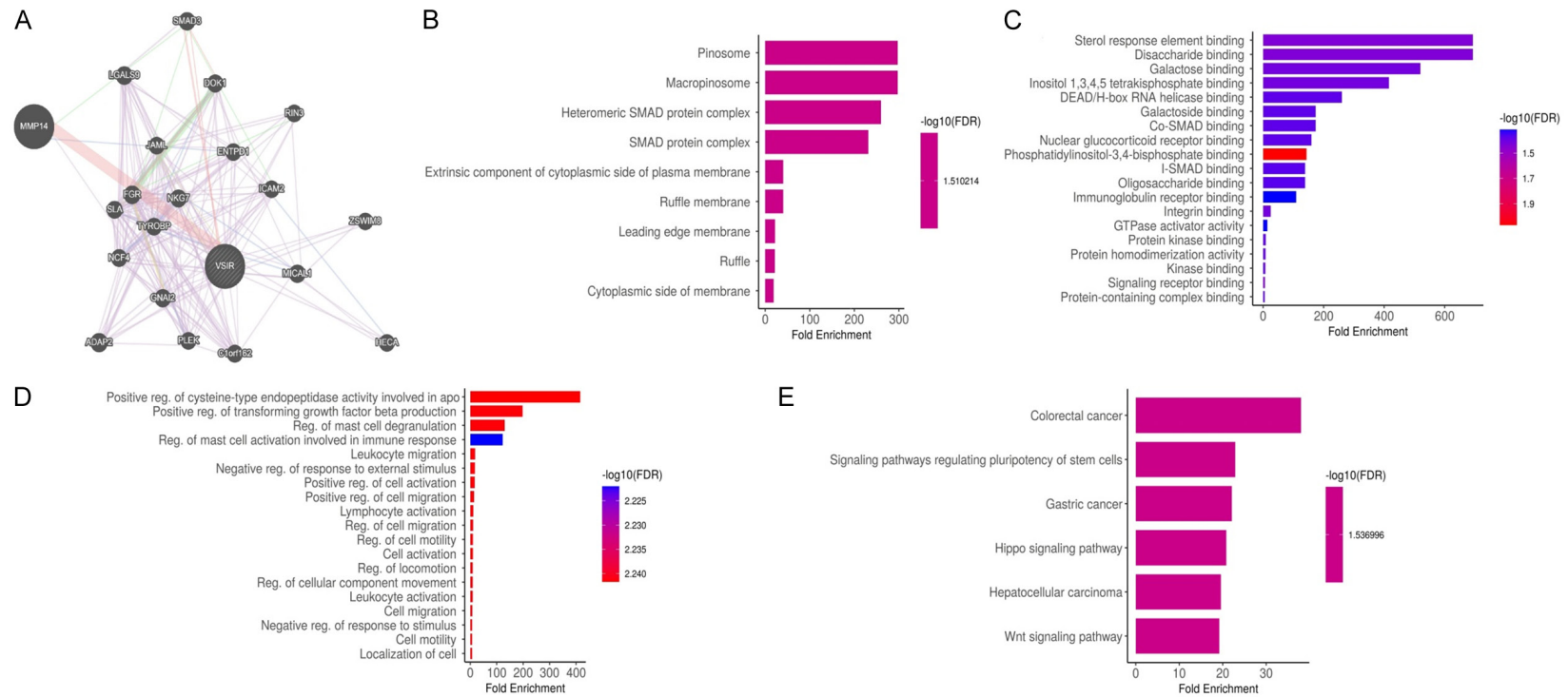


Figure 6. Gene enrichment analysis related to V-set immunoregulatory receptor (VSIR). A. Protein-Protein Interaction (PPI) network of VSIR-related proteins. B. Cellular Component (CC) terms associated with VSIR-related gene enrichment. C. Molecular Functions (MF) terms linked to VSIR-related gene enrichment. D. Biological Processes (BP) terms associated with VSIR-related gene enrichment. E. Kyoto Encyclopedia of Genes and Genomes (KEGG) terms related to VSIR-associated dysregulated pathways. *P*-value < 0.05.

VSIR as a pan-cancer marker

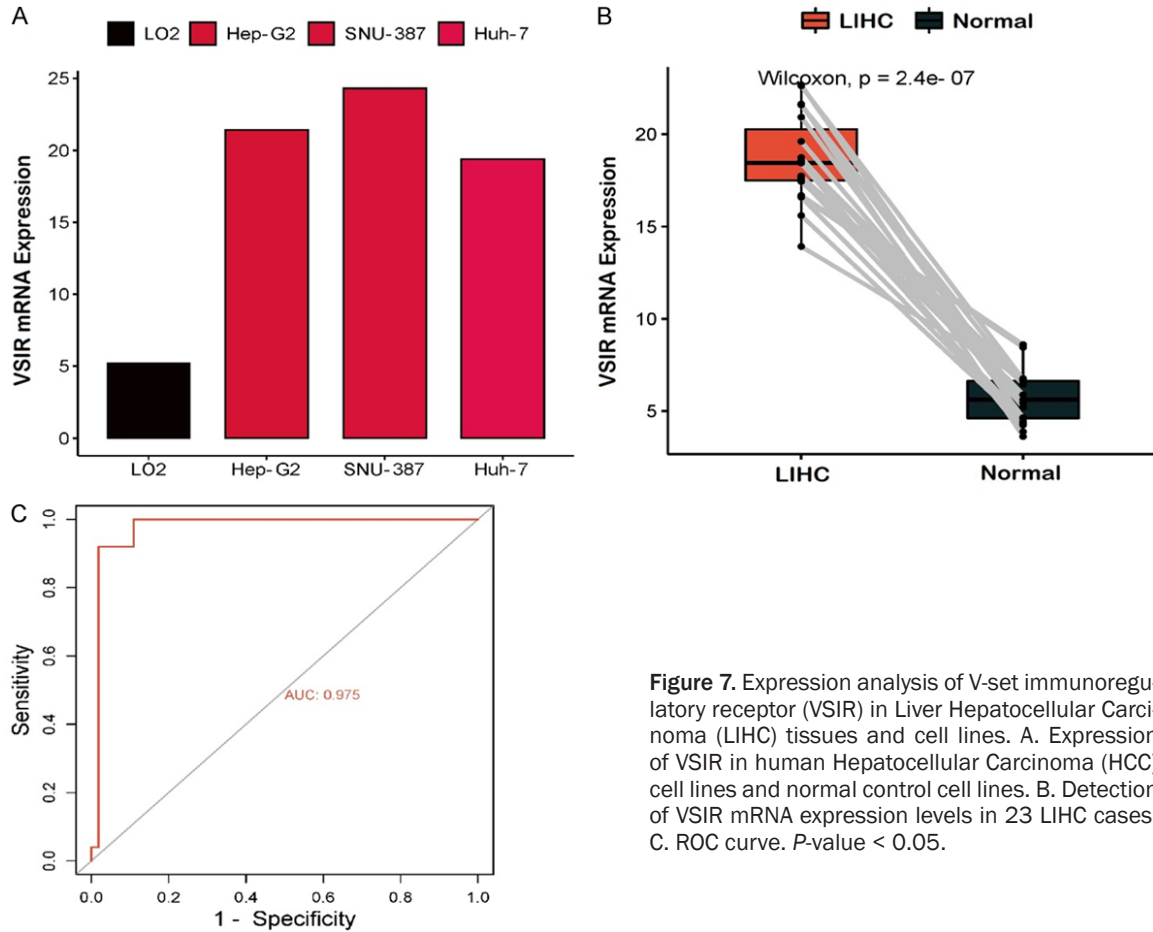


Figure 7. Expression analysis of V-set immunoregulatory receptor (VSIR) in Liver Hepatocellular Carcinoma (LIHC) tissues and cell lines. A. Expression of VSIR in human Hepatocellular Carcinoma (HCC) cell lines and normal control cell lines. B. Detection of VSIR mRNA expression levels in 23 LIHC cases. C. ROC curve. P -value < 0.05.

validate the oncogenic role of VSIR, reinforcing its significance in LIHC.

Discussion

This study explored the expression profile and prognostic significance of VSIR across various types of cancer. Elevated levels of VSIR were observed in ESCA and LIHC, correlating with unfavorable prognoses. Additionally, our findings highlight the outstanding predictive potential of VSIR in these cancers, paving the way for further investigation into the role of VSIR in tumor immunity within these specific contexts.

In prior research, VSIR has been identified to play dual roles in tumor immunity, exhibiting both negative and positive effects. Up-regulation of VSIR on tumor-infiltrating myeloid cells has been linked to the promotion of tumor growth by suppressing T cell immunity [37, 38]. Notably, the use of a specific antibody that neutralizes VSIR has proven effective in inhibiting tumor growth in mouse models [39, 40].

Divergent outcomes have been reported in previous studies regarding the clinical implications of VSIR levels. Higher VSIR levels have been associated with improved clinical prognosis in epithelioid mesothelioma cancer [41], non-small-cell lung cancer [42], and esophageal adenocarcinoma [42]. However, Kuklinski et al. discovered that increased VSIR levels were linked to unfavorable disease-specific survival (DSS) in primary cutaneous melanoma [43].

Examining distinct cancer stages revealed a significant correlation between VSIR expression and varying stages, suggesting its potential role in disease progression. The observed decrease in VSIR promoter methylation levels in ESCA and LIHC tumors compared to adjacent normal tissues underscores the involvement of methylation patterns in oncogenesis, possibly acting as either promoters or inhibitors of tumor formation. Mutational analysis of VSIR across ESCA and LIHC samples revealed a 0% mutational frequency, indicating that VSIR remains

unaltered at the genetic level in these cancers. This information is crucial, as genetic mutations often play a pivotal role in disrupting the expression of key genes and contributing to cancer development.

In the development of tumor formation, growth, metastasis, and recurrence, tumor-infiltrating inflammatory cells play a crucial role. Tregs, an essential component for maintaining immune homeostasis, contribute significantly to tumor immunity by inhibiting the activation and differentiation of CD4⁺ helper T cells and CD8⁺ cytotoxic T cells [44]. During the course of the tumor immune response, Tregs derived from ordinary T cells release immunosuppressive factors such as TGF- β , IL-10, and IL-35, leading to the inhibition of antitumor immunity and the facilitation of tumor formation and progression [45]. Efforts to target Cancer-Associated Fibroblasts (CAFs) have recently emerged as a promising avenue for enhancing cancer therapy. CAFs exert regulatory control over cancer metastasis through intricate mechanisms, including ECM remodeling, induction of growth factors, impact on drug resistance and immunotherapy response, modification of interactions with cancer cells, and infiltration of inflammatory cells [46].

The findings of this study reveal a positive correlation between VSIR expression and various immune cell types in both ESCA and LIHC. The association extends to B cells, CD8⁺ T cells, CD4⁺ T cells, macrophages, neutrophils, and dendritic cells. This indicates an intricate interplay between VSIR and the immune microenvironment in these specific cancer types. Such positive correlations may have implications for understanding the role of VSIR in modulating immune responses and suggest its involvement in shaping the tumor microenvironment.

Nevertheless, the limitations of this study primarily stem from the absence of additional experiments such as immunohistochemistry and western blot-based techniques. The association between the low expression of VSIR in most malignancies and a spectrum of contentious immunological and survival outcomes necessitates further exploration to elucidate the exact mechanisms involved. To understand the underlying mechanisms of VSIR across various cancer types, it is imperative to perform additional experimental validation.

Conclusion

This study revealed the diagnostic and prognostic roles of VSIR in esophageal and liver cancers. Notably, VSIR expression correlates significantly with cancer stages, highlighting its potential in disease progression. Promoter methylation analysis underscores the intricate link between epigenetics and oncogenesis. Subcellular localization prediction shows that VSIR is predominantly in the nucleoplasm, suggesting involvement in nuclear processes. Negative correlations with TMB and MSI emphasize its role as a biomarker in immune response and genomic stability.

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Disclosure of conflict of interest

None.

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VSIR as a pan-cancer marker

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